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Role of Protein Kinase C Isoforms in Phorbol Ester-induced Vascular Endothelial Growth Factor Expression in Human Glioblastoma Cells*

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Aberrant expression of the potent angiogenic cytokine, vascular endothelial growth factor (VEGF), has been demonstrated to be associated with most human solid tumors. Both transcriptional and post-transcriptional mechanisms have been shown to modulate VEGF expression in a multitude of cell types. Here we report that when protein kinase C (PKC) pathways were activated in human glioblastoma U373 cells by phorbol 12-myristate 13-acetate (PMA), VEGF mRNA expression was up-regulated via a post-transcriptional mRNA stabilization mechanism. PMA treatment exhibited no increase in VEGF-specific transcriptional activation as determined by run-off transcription assays and VEGF promoter-luciferase reporter assays. However, PMA increased VEGF mRNA half-life from 0.8 to 3.6 h which was blocked by PKC inhibitors but not by protein kinase A or cyclic nucleotide-dependent protein kinase inhibitors. When U373 cells were transfected with antisense oligonucleotide sequences to the translation start sites of PKC-α, -β, -γ, -δ, -ϵ, or -ζ isoforms, both PKC-α and -ζ antisense oligonucleotides showed substantial inhibition of PMA-induced VEGF mRNA. In addition, overexpression of PKC-ζ resulted in a strong constitutive up-regulation of VEGF mRNA expression. This study demonstrates for the first time that specific PKC isoforms regulate VEGF mRNA expression through post-transcriptional mechanisms.

The regulation of vascular endothelial growth factor (VEGF)[1] expression by human tumor cells has been implicated as a key factor in human tumorigenic and metastatic potential (1, 2). VEGF expression has been shown to be controlled by environmental factors such as the limited availability of oxygen or glucose (2). Alternatively, growth factors like platelet-derived growth factor and acidic/basic fibroblast growth factor or cytokines like tumor necrosis factor-α and transforming growth factor family members also have been shown to stimulate VEGF synthesis (2). It is clear that VEGF expression is tightly regulated by both transcriptional and post-transcriptional mechanisms (2, 3); however, the signal transduction pathways that regulate these mechanisms remain largely unknown.

The protein kinase C (PKC) family consists of 12 phospholipid-dependent serine/threonine kinases that mediate signals from the cell surface to the nucleus and play key roles in cellular signaling pathways (4, 5). These isoenzymes have been grouped into three functional classes based on the structures of their regulatory domains and their requirements for activation by phosphatidylserine, calcium, and diacylglycerol (DAG) (4, 5). The first class, the conventional PKCs (cPKCs, isoforms α, βI, βII, and γ), require Ca^{2+} and DAG to be activated. The second class, the novel PKCs (nPKCs, isoforms δ, ϵ, η, and θ), are Ca^{2+}-independent. The third class, the atypical PKCs (aPKCs, isoforms ζ, λ, τ, and μ), are dependent on phosphatidylserine but not Ca^{2+} or DAG. PKC isoenzymes exhibit distinct tissue distribution patterns, and most cell types express PKC-α, -δ, and -ζ, whereas other isoforms are restricted to some specific tissues (4, 5). Several reports have indicated that activation of PKC by the tumor promoter, phorbol 12-myristate 13-acetate (PMA), up-regulates VEGF expression in a variety of cell types including astrocytes, fibroblasts, umbilical vascular endothelial cells, and smooth muscle cells (6–9). However, the relative importance of individual PKC isoforms to transcriptional and post-transcriptional regulation of VEGF expression remains unclear.

Human glioblastomas are one of the most rapidly growing angiogenic tumors corresponding to high mortality and morbidity rates. Here we show that when U373 glioblastoma cells were treated with PMA, VEGF mRNA expression was up-regulated through a post-transcriptional mRNA stabilization mechanism but not transcriptional activation. We show that PKC-α and -ζ are involved in the induction of VEGF expression as defined by antisense oligonucleotide inhibition and overexpression studies. This study indicates that PKC isoform activation, especially PKC-α and -ζ, are important upstream events resulting in increased VEGF mRNA stabilization.

MATERIALS AND METHODS

Antibodies, Oligonucleotides, and Kinase Inhibitors—Monoclonal antibodies to different PKC isoforms were purchased from Transduction Laboratories (Lexington, KY). The chicken anti-hVEGF polyclonal antibody was raised to human baculovirus-expressed human VEGF165. The mouse anti-hVEGF monoclonal antibody was purchased from R & D Systems (Minneapolis, MN). All of the antisense PKC oligonucleotides to PKC-α, -β, -γ, -δ, -ϵ, and -ζ were synthesized as phosphorothioate derivatives from Genemed Synthesis (San Francisco, CA). Seven

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; AS, antisense oligonucleotides; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); nPKC, novel PKC; cPKC, conventional PKC; aPKC, atypical PKC; h, human.

different kinase inhibitors were used and purchased from Calbiochem as follows: protein kinase C inhibitors staurosporine (highly specific inhibitor of PKC) (10), bisindolemaleimide I (high selectivity for PKC-α, -β, -γ, -δ and -ϵ) (11), Go6976 (high selectivity for PKC-α and -β) (12), and calphostin C (highly specific inhibitor of PKC) (13); protein kinase A inhibitor H89 (14); serine/threonine kinase inhibitor, H7 (15); and cyclic nucleotide-dependent protein kinase inhibitor, H8 (16).

Cell Lines and Culture Conditions—Human glioblastoma cell line, U373-MG, was routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin. Cells were cultured under either normoxic conditions (5% CO_2, 21% O_2, 74% N_2) or hypoxic conditions (5% CO_2, 2% O_2, 93% N_2) in a humidified triple gas incubator (Heraeus model 6060, Hanau, Germany) at 37 °C.

Western Blot and Luciferase Assays—Cytoplasmic extracts were obtained as described (17). Briefly, U373 cells were washed three times in ice-cold PBS before lysis with buffer containing 50 mM Hepes, pH 7.5, 1% Triton X-100, 10 mM sodium pyrophosphate, 150 mM NaCl, 100 mM NaF, 0.2 mM $NaVO_4$, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, and 5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma). The cytoplasmic extract was collected after centrifugation at 14,000 × *g* for 15 min, denatured under reducing conditions, and proteins separated in 8% SDS-PAGE. Western blots were performed using a 1:1000 dilution of anti-PKC monoclonal antibodies followed by a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech) in a blocking buffer containing 1% bovine serum albumin and 0.1% Tween 20 in Tris-buffered saline, pH 7.4. The blots were then developed with ECL system (Amersham Pharmacia Biotech). For luciferase reporter assays, stably transfected U373 cells containing the VEGF promoter construct (−1226 to +298 base pairs upstream of luciferase reporter) (18) were lysed with passive lysis buffer, and luciferase activity was measured with a Luciferase Assay kit following the supplier's instructions (Promega, Madison, WI).

Enzyme-linked Immunosorbent Assay—In a 96-well plate (Dynatech Laboratories, Chantilly, VA), 100 μl of chicken anti-hVEGF polyclonal antibody (0.5 μg/ml in PBS) was coated overnight at 4 °C. The plate was then blocked with blocking buffer (0.25% bovine serum albumin, 0.05% Tween 20, and 1 mM EDTA in PBS) for 1 h at 37 °C. Conditioned media (100 μl) or recombinant hVEGF standard (R & D System) was added and incubation continued for another 2 h at 37 °C. Mouse anti-hVEGF monoclonal antibody (R & D System) was added (100 μl of 0.5 ng/ml) to each well after washing the plate with PBS, 0.05% Tween 20 solution and then incubated at 37 °C for 2 h. Detection antibody, a peroxidase-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), was incubated at 37 °C for 2 h, and the plates were washed again with PBS, 0.05% Tween 20. MBT substrate (Kirkegaard & Perry Laboratories) was used to develop color and quantitated with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

Transcription Run-off—Transcription run-off assay were performed as described earlier (17). Briefly, U373 cell nuclei were collected in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.35% sucrose, and 0.5% Nonidet P-40) and centrifuged for 3 min at 500 × *g*. Nuclei were then suspended in reaction buffer (5 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 0.5 mM rATP/CTP/GTP, 0.7 μM rUTP, 2.5 mM dithiothreitol, and 5 units RNasin) in the presence of 100 μCi of [α-^{32}P]UTP and incubated at 30 °C for 23 min. Total RNA was extracted by the addition of 40 μg of glycogen carrier and 0.8 ml of Trizol reagent. The radiolabeled RNA pellets were collected after phenol/chloroform extraction and isopropyl alcohol precipitation (0.5 volume) and dissolved in 10 mM β-mercaptoethanol solution. Slot blots used for RNA hybridizations were prepared with VEGF, 36B4 and glyceraldehyde-3-phosphate dehydrogenase cDNAs (5 μg/slot). The cDNAs were denatured with 100 mM NaOH at 100 °C for 10 min, neutralized with NH_4OAc to 1 M final concentration, and directly blotted onto nylon membranes (ICN, Costa Mesa, CA) using a slot blot apparatus (Schleicher & Schuell). Membranes were subsequently washed twice with 2× SSC, baked at 80 °C for 15 min, and prehybridized for 6 h at 65 °C in hybridization buffer ([50 mM PIPES, pH 6.5, 50 mM sodium phosphate, pH 7.0, 20 mM NaCl, 5% SDS, 2.5 mM EDTA, and 50 μg/ml denatured salmon sperm DNA). Hybridizations were performed with equal amounts of labeled nuclear RNA (1×10^6 cpm/ml) in hybridization buffer for 20 h at 65 °C. Blots were then washed once at room temperature for 20 min and twice at 55 °C for 20 min in 1% SDS, 1× SSC. Blots were then exposed to Kodak MR film. VEGF mRNA was normalized to 36B4 expression using PhosphorImager analysis (Molecular Dynamics).

Transient Transfection, mRNA Extraction, and Northern Analysis—Antisense oligonucleotides to PKC-α, -β, -γ, -δ, -ϵ, and -ζ (0.1, 0.5, or 1 μM) or FLAG epitope-tagged PKC-ζ overexpression vector (19) were transfected into U373 cells using LipofectAMINE (Life Technologies, Inc.) following the supplier's instructions. Total RNA was extracted by using RNeasy RNA extraction kit (Qiagen, Chatsworth, CA). Northern blot was performed as described previously (17). Briefly, equal amounts of total RNA (10 μg/lane) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde. RNA was blotted by capillary transfer to a nylon membrane (NEN Life Science Products) in 10× SSC. Blots were cross-linked with UV Stratalinker (Stratagene), baked at 80 °C for 15 min, and prehybridized for 2–4 h at 65 °C. Hybridization was carried out overnight at 65 °C with [α-^{32}P]dCTP-labeled human VEGF165 *Acc*I/*Nco*I fragment (823 base pairs) encompassing the coding region and 330 base pairs of 3′-untranslated region. A ribosome-associated protein cDNA, 36B4, was used as a control. Probes were prepared by the random-primed synthesis method using the Multiprime Kit (Amersham Pharmacia Biotech). Blots were washed at high stringency (1% SDS, 1× SSC at 60 °C) and exposed to Kodak MR film. VEGF mRNA was normalized to 36B4 expression using PhosphorImager analysis.

Determination of VEGF mRNA Half-life—The transcription inhibitor actinomycin D (5 μg/ml) was added to U373 cells and total RNA isolated at various time intervals (30 min and 1, 2, and 4 h). Total RNA was analyzed by Northern blot, and VEGF signal was quantified by PhosphorImager analysis. The VEGF signal was normalized to ribosomal associated protein 36B4 signal. Least squares regression analysis of the resulting line was performed (Kaleidagraph, Synergy Software, Reading, PA), and half-life values were determined from the regression curves.

In Vitro PKC Activity Assay—The *in vitro* PKC activity assays were performed as described (20). Briefly, U373 cells were washed three times in ice-cold PBS followed by lysis in 1% Triton X-100 lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.2 mM $NaVO_4$, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. The lysate was incubated with rabbit anti-human PKC-α, -δ, or -ζ polyclonal antibody (Life Technologies, Inc.) for 2 h at 4 °C followed by another 2-h incubation with 20 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C. The immunoprecipitates were washed six times with the lysis buffer containing 0.5 M NaCl. The immunocomplexes were suspended in 20 μl of kinase buffer (35 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$, and 1 mM phenylphosphate) containing 2 μg of myelin basic protein (MBP, Sigma) and 5 μCi of [γ-^{32}P]ATP and incubated for 30 min at 30 °C. Reactions were stopped by the addition of SDS-PAGE sample buffer. Samples were denatured and separated by 12% SDS-PAGE followed by exposure to Kodak MR film. MBP and ^{32}P-MBP signals were quantified by densitometer and PhosphorImager analysis, respectively.

RESULTS

Protein Kinase C Activation Potently Induces VEGF Expression—To determine the relative contribution of protein kinase C (PKC) activation to the induction of VEGF mRNA expression in human glioblastoma U373 cells, phorbol 12-myristate 13-acetate (PMA) was used to activate PKCs, and steady-state VEGF mRNA levels were determined by Northern blot. As shown in Fig. 1*A*, VEGF mRNA levels were increased 5.8-fold with 4 h PMA treatment when compared with U373 cells without PMA activation. The 4-h PMA-induced VEGF mRNA expression was approximately half of the 18-h hypoxia-induced signal (Fig. 1*A*). In a time course study of PMA treatment (100 nM), induction of VEGF mRNA can be observed at 1 h which increased to a maximum level at 8 h (Fig. 1*B*) and then slowly decreased afterward (data not shown). Furthermore, this response was dependent upon cell confluence, showing a PMA induction of VEGF mRNA expression that varied from 3- to 10-fold at 4 h (data not shown). Taken together, these results suggest that PMA strongly up-regulates VEGF mRNA expression in U373 cells, and the activation requires prolonged PMA treatment with maximum VEGF mRNA levels reached at 8 h.

To study whether PMA up-regulation of VEGF mRNA expression correlates to VEGF protein synthesis and secretion, we performed enzyme-linked immunosorbent assay analysis of U373-conditioned media with and without the addition of PMA for 4 h. The conditioned media of control cells contained 0.313 ± 0.076 ng/ml VEGF, and PMA-treated cells contained

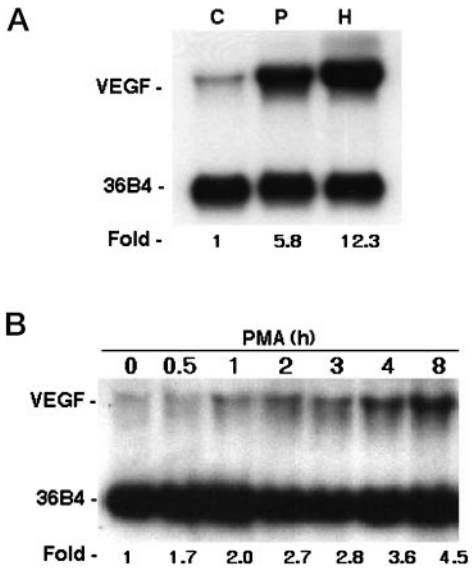

FIG. 1. **PMA up-regulates VEGF mRNA expression.** *A*, VEGF mRNA expression in U373 cells. U373 cells were cultured alone (*C*), treated with PMA (100 nM) for 4 h (*P*), or cultured under hypoxic conditions (3% O_2) for 18 h (*H*). The fold induction of VEGF mRNA over control (*C*) was calculated after normalization to the ribosome-associated protein, 36B4, from each lane (*Fold*). *B*, time course studies of PMA induction of VEGF mRNA expression. U373 cells were treated with PMA (100 nM) for various times as indicated. Total mRNA was extracted and analyzed for VEGF mRNA by Northern blot. The ribosome-associated protein, 36B4, was used as mRNA control. Fold induction of VEGF mRNA without PMA treatment (*0*) was calculated after normalization to the 36B4 mRNA from each lane (*Fold*).

0.79 ± 0.124 ng/ml VEGF. This is a 2.5-fold induction of VEGF secretion in only 4 h and is statistically significant in a Student's *t* test ($p \leq 0.0047$). Thus, increases observed in VEGF mRNA levels with PMA treatment correlate well with increases in VEGF protein synthesis and secretion.

The PKC isoforms α, δ, and ζ, representing cPKC, nPKC, and aPKC classes respectively, are universally expressed (4). Studies have shown that prolonged PMA treatment can repress cPKCs and nPKCs but not aPKCs synthesis (4, 21). Thus, it was of interest to determine the effect of PMA on the three universally expressed PKC isoforms in U373 cells. Fig. 2*A* is a representative Western blot showing PMA regulation of PKC synthesis. Both PKC-α and -δ protein levels decreased over time with PMA treatment, and PKC-α reached almost undetectable levels after 4 h of PMA exposure. In a similar but less effective manner, PKC-δ decreased by 50% with 8 h PMA treatment. In contrast, PKC-ζ protein showed a substantial increase and almost doubled after 30 min and remained elevated up to at least 8 h (Fig. 2*A*) and then slowly decreased afterward (data not shown). By using an *in vitro* PKC activity assay (Fig. 2, *B* and *C*), specific immunoprecipitation followed by phosphorylation of myelin basic protein (MBP), PKC-α activity reached a maximum of 141% over control at 15 min PMA and then decreased sharply over 4 h PMA treatment to 53% of control. PKC-δ activity showed a slower induction than PKC-α and reached maximum of 121% over control at 30 min PMA and then slowly decreased over the time of PMA treatment to 91% of control at 4 h PMA. In contrast, PKC-ζ activity gradually increased during PMA treatment and remained elevated up to 4 h PMA with a maximum of 158% over control at 4 h PMA exposure. These results suggest that PMA differentially regulates not only PKC isoform synthesis but also selectively modulates their activity in U373 cells. A transient, rapid activation of PKC-α activity, followed by stable up-regulation of PKC-ζ activity over the 4-h PMA treatment, suggests that a constant total PKC activity may be necessary to functionally up-regulate VEGF mRNA expression in U373 cells.

Protein Kinase C Inhibitors Suppress Phorbol Ester-induced VEGF mRNA Expression—In order to study further the involvement of PKC isoforms to the phorbol ester-induced VEGF mRNA expression, we employed several kinase inhibitors at active concentrations and studied PMA-induced VEGF mRNA levels. A representative result is shown in Fig. 3; the broad PKC inhibitors, staurosporine and calphostin C, strongly decreased PMA-induced VEGF mRNA expression. However, PKC inhibitors selective to cPKCs (Go6967) or to both cPKCs and nPKCs (bisindolemaleimide I) were less effective in repressing PMA-induced VEGF mRNA. This suggests that the aPKC isoforms may also play a role in the PMA-induced VEGF mRNA expression. Application of a broad serine/threonine kinase inhibitor H7 showed significant reduction of PMA-induced VEGF mRNA expression, whereas the protein kinase A inhibitor H89 did not show any suppression. This indicates that protein kinase A is not involved in the PMA activation of VEGF mRNA expression, and inhibition of VEGF mRNA accumulation by the serine/threonine kinase inhibitor H7 is likely mediated through PKC. The cyclic nucleotide-dependent protein kinase inhibitor, H8, interestingly showed an increase in VEGF mRNA levels, indicating that blocking of cyclic nucleotide-dependent protein kinase may favor PMA activation pathways and VEGF mRNA accumulation. Taken together, these results suggest that the induction of VEGF mRNA expression by PMA is mainly mediated through a cascade of PKC isoform activation, including aPKCs.

PKC Activation Induces VEGF mRNA Steady-state Levels That Are Independent of Transcriptional Activation—To determine whether increased VEGF mRNA expression by PMA is specifically due to transcriptional activation, two studies were performed. First, VEGF mRNA transcription in U373 cells was measured by run-off transcription assay. As shown in Fig. 4*A*, only hypoxic conditions showed measurable activation of VEGF mRNA transcription, whereas 4 h of PMA treatment did not when compared with control. This experiment has been repeated three times with identical results. Second, U373 cells were transfected and selected for a stable integrated human VEGF promoter construct (−1226 to +298 base pairs) upstream of the luciferase reporter gene. These cells were then treated with PMA and analyzed for luciferase activity. As shown in Fig. 4*B*, there was little or no increase in luciferase activity from 1 to 12 h PMA treatment, whereas 12 h of hypoxia showed a 2.5-fold transcriptional activation. Thus, the VEGF promoter/reporter construct is functional and responsive to hypoxia, and PMA does not activate VEGF promoter transcription. Taken together, these results suggest that PMA-induced VEGF mRNA accumulation is not mediated through transcriptional activation as is the case for hypoxic stress.

Phorbol Ester Induces VEGF mRNA Stability That Is Reversible with Staurosporine Treatment—To determine whether post-transcriptional regulation plays a crucial role in the PMA induction of VEGF mRNA expression, we treated U373 cells with PMA for 3 h prior to the addition of the transcription inhibitor actinomycin D, and we determined the VEGF mRNA half-life with quantitative Northern blot analysis. As shown in Fig. 5, VEGF mRNA half-life increased from 0.8 to 3.6 h after treatment of U373 cells with PMA. Since the broad PKC inhibitor staurosporine showed a strong inhibition of PMA up-regulation of VEGF mRNA expression (Fig. 3), the inhibitor was also added during the course of PMA treatment, and VEGF mRNA stability was measured. As shown in Fig. 5, staurosporine limited the PMA-induced VEGF mRNA half-life to 1.3 h (Fig. 5). This result suggests that increased VEGF mRNA

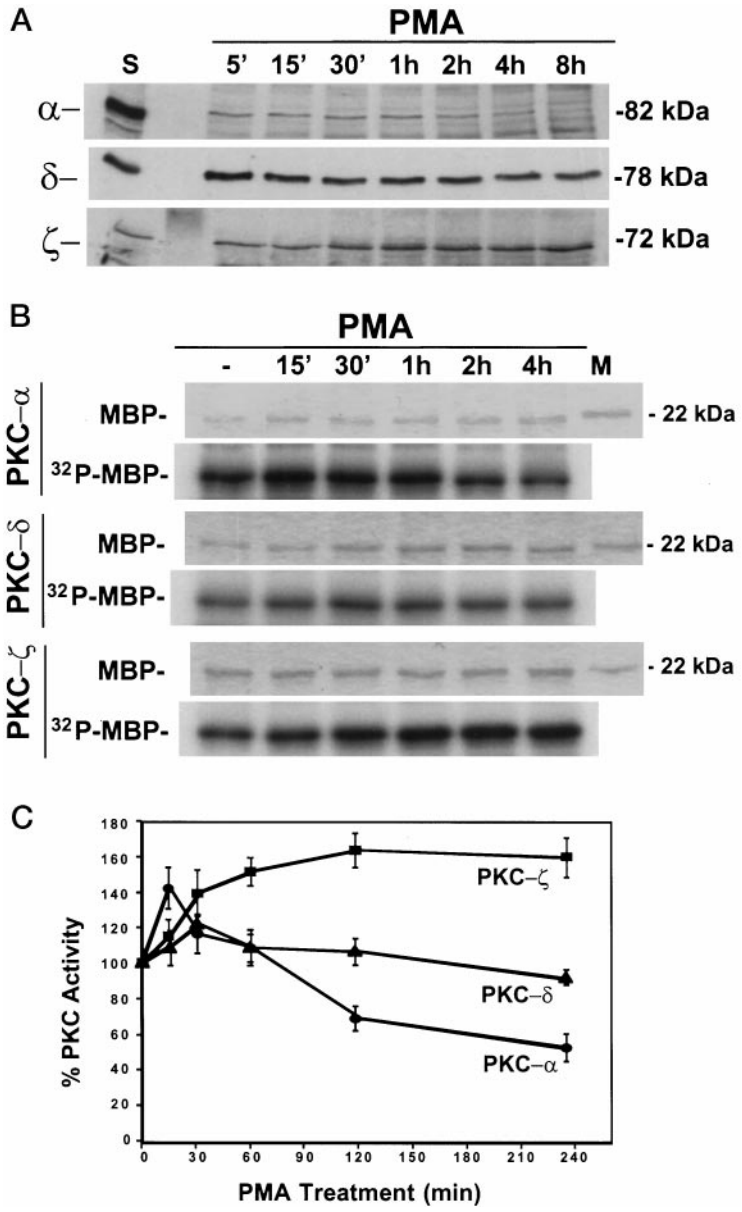

FIG. 2. **Differential regulation of PKC expression and PKC activity by PMA.** *A*, effect of PMA on PKC isoform synthesis. U373 cells were treated with PMA (100 nM) for various times as indicated. Total cellular proteins were extracted, and PKC-α, -δ, and -ζ protein levels were detected by Western blot. *S* indicates standard PKCs from Jurkat cell lysate. *B*, representative *in vitro* PKC activity after PMA treatment. Total cellular proteins were prepared after different time intervals of PMA (100 nM) treatment, PKC-α, -δ or -ζ immunoprecipitated, and *in vitro* phosphorylation activity tested with 21-kDa myelin basic protein (*MBP*) as a substrate and analyzed ^{32}P-MBP after separation in 12% SDS-PAGE. *M* indicates 22-kDa protein marker. *C*, percent PKC activity over control (−) was calculated after normalization to Coomassie Blue-stained MBP from each lane. MBP and ^{32}P-MBP signals were quantified by densitometer and PhosphorImager analysis, respectively. Results are presented as the means ± S.D. of two independent experiments.

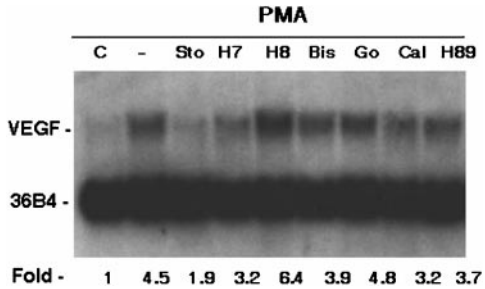

FIG. 3. **Inhibition of PMA-induced VEGF mRNA expression by kinase inhibitors.** U373 cells were cultured alone (*C*) or treated with PMA for 4 h in the absence or presence of kinase inhibitors; staurosporine (100 nM; *Sto*), H7 (25 μM), H8 (25 μM), bisindolemaleimide I (100 nM; *Bis*), Go6976 (10 nM; *Go*), calphostin C (100 nM; *Cal*), and H89 (1 μM). Total RNA was extracted, and VEGF mRNA was detected by Northern blot analysis. The ribosome-associated protein probe, 36B4, was used as control. Fold induction of VEGF mRNA over control (*C*) was calculated after normalization to the ribosome-associated protein mRNA, 36B4, from each lane (*Fold*).

expression by PMA is regulated predominantly through a post-transcriptional mechanism, and the activation of PKC plays an important role in this mechanism.

PKC-α and -ζ Antisense Oligonucleotides Block the PMA Induction of VEGF mRNA Expression—Of the 12 PKC isoforms identified to date, 8 PKC isoforms were detected in the U373 cells by Western blot analysis, and the PKC-βI, -βII, -η, and -θ isoforms were excluded (data not shown). To delineate the PKC isoforms contributing to PMA-induced VEGF mRNA stability, six antisense (AS) oligonucleotides were selected from the three subclasses of PKC isoforms to access their individual functions. The AS oligonucleotides were designed against the translation start site of each PKC isoform (Fig. 6*A*). To improve intracellular stability and effectiveness, the AS oligonucleotides were synthesized as phosphorothioate derivatives. To ensure the AS oligonucleotides were effective in repressing their respective PKC isoform expression, we performed Western blot analysis on transfected cells. A representative result is shown in Fig. 6*B*, all six of the AS oligonucleotides (0.5 μM) substantially reduced their specific PKC protein levels. PKC-α and -ζ AS oligonucleotides decreased their respective protein levels approximately 80–90%, whereas PKC-γ, -δ and -ε AS oligonucleotides repressed their isoforms by about 40–50%.

Given the effective reduction of PKC-α and -ζ isoforms by their specific AS oligonucleotides, we examined whether down-

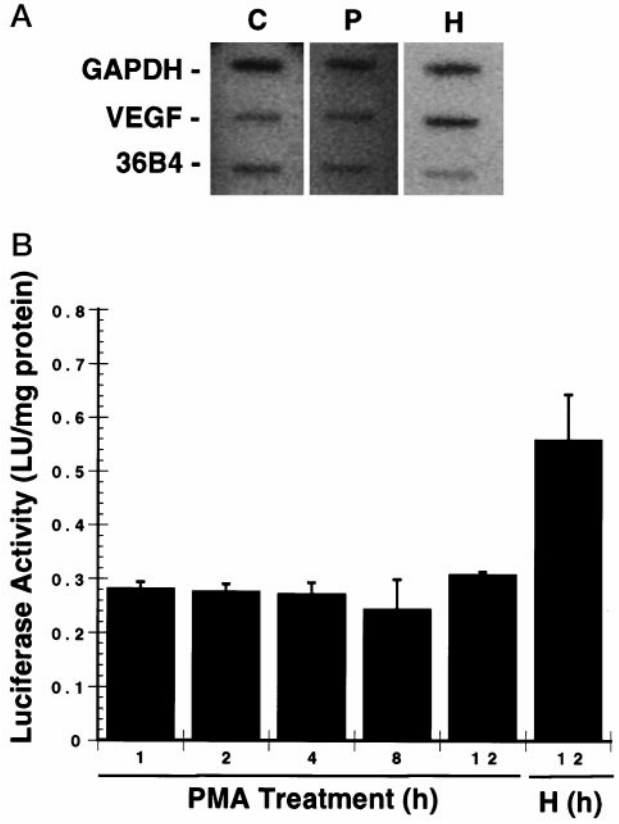

FIG. 4. **PMA-induced VEGF mRNA expression is not transcriptionally activated.** *A*, transcription run-off assays. U373 cells were cultured alone (*C*) or treated with PMA for 4 h (*P*) or hypoxia (3% O_2) for 8 h (*H*). Total radiolabeled RNA (10^6 cpm/ml) obtained from nuclear run-off reactions was hybridized to denatured cDNA templates encoding GADPH, VEGF, and 36B4. *B*, luciferase reporter assays. The stably transfected U373 cells containing the VEGF promoter (−1226 to +298 base pairs upstream of luciferase) were either treated with PMA (100 nM) for various times as indicated or cultured under hypoxia for 12 h (*H*) in triplicate. Luciferase activities (mean ± S.D.) were measured as described under "Materials and Methods."

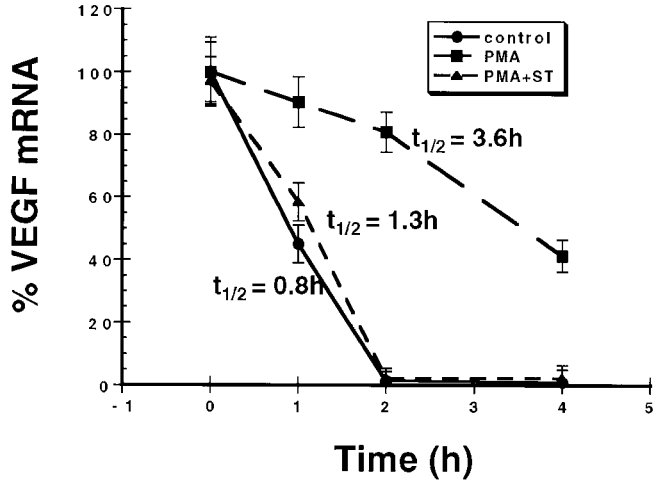

FIG. 5. **Staurosporine suppresses PMA-induced VEGF mRNA stability.** U373 cells were cultured alone (*control*) or treated with PMA for 3 h (*PMA*) in the absence or presence of staurosporine (*PMA+ST*) in a triplicate experiment. The cells were then treated with actinomycin D (5 μg/ml) for various time intervals (0, 1, 2, and 4 h), and VEGF mRNA levels were detected by Northern blot analysis. VEGF mRNA signal was quantified by PhosphorImager analysis and normalized with the ribosome-associated protein, 36B4 mRNA. Values are expressed as mean ± S.D. VEGF mRNA signal without actinomycin D treatment was defined as 100%, and VEGF mRNA signals after actinomycin D treatment were calculated as percent VEGF mRNA remaining.

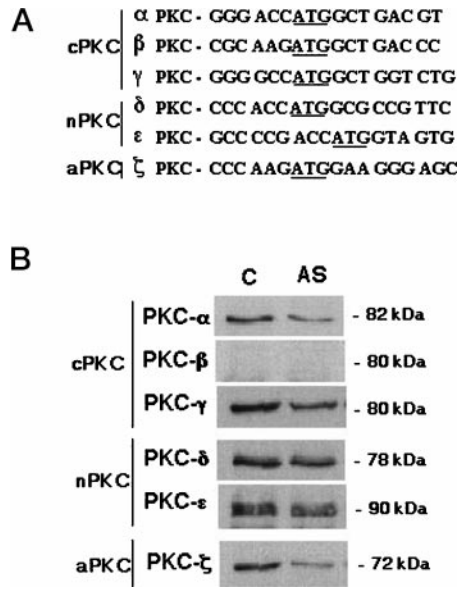

FIG. 6. **Effect of antisense oligonucleotides on PKC synthesis.** *A*, antisense oligonucleotide sequences to translation start sites of individual PKC isoforms. *B*, Western blot detection of PKC isoform synthesis. U373 cells were transiently transfected with Lipofectin reagent in the absence (*C*) or presence of 0.5 μM antisense (*AS*) oligonucleotides to PKC-α, -β, -γ, -δ, -ε, or -ζ. After 18 h recovery, cytoplasmic extracts were obtained, and the respective PKC proteins were detected by Western blot.

regulation of either of these PKC isoforms would interfere with PMA-induced VEGF mRNA expression by quantitative Northern blot. As a control, cells were transfected with AS oligonucleotide to PKC-β, which is not expressed by U373 cells. As shown in Fig. 7*A*, cells transfected in triplicate with PKC-α or -ζ AS oligonucleotide showed a dose-dependent reduction of PMA-induced VEGF mRNA level, whereas the control PKC-β AS oligonucleotide was ineffective. With the exception of the lowest concentration (0.1 μM), both 0.5 and 1 μM AS oligonucleotide transfection to PKC-α or -ζ showed a statistically significant reduction in PMA-induced VEGF mRNA expression (Fig. 7*A*, $p < 0.05$). Fig. 7*B* is a representative Northern blot showing a dose-dependent repression of PMA-induced VEGF mRNA level by PKC-α or -ζ AS oligonucleotide transfection. The U373 cells transfected with PKC-δ or -ε AS oligonucleotides did not demonstrate any significant repression of PMA-induced VEGF mRNA expression when performed in triplicate (data not shown). Taken together, these results suggest that both PKC-α and -ζ isoforms are crucial mediators in the PMA activation of VEGF mRNA expression in U373 cells.

Studies have suggested that PKC isoforms may regulate their activity through direct or indirect cross-talk (22). Given the effectiveness of transient transfection of either PKC-α or -ζ AS oligonucleotides for limiting the PMA induction of VEGF mRNA expression and the overlap in PMA-induced activity between the two isoforms, it was of interest to detect a possible cross-talk between the two isoforms in U373 cells. A representative Western blot is shown in Fig. 8*A*, identifying that the transfection of U373 cells with PKC-α AS oligonucleotide not only blocked its own protein synthesis but also significantly reduced PKC-ζ protein levels. Conversely, AS oligonucleotide to PKC-ζ also strongly inhibited PKC-α protein levels after the transfection. In contrast, the control AS oligonucleotide to PKC-β did not show any significant effect on the synthesis of either PKC-α or -ζ. By using an *in vitro* PKC activity assay to access PKC isoform activities with AS oligonucleotide transfection (Fig. 8*B*), the reduction of PKC-α protein level by both

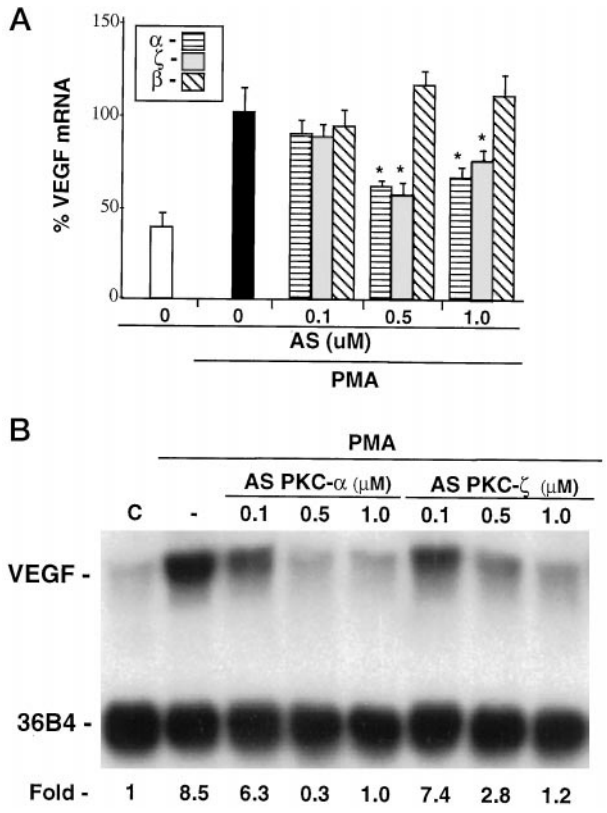

FIG. 7. **PKC-α and -ζ antisense oligonucleotides block PMA-induced VEGF mRNA expression.** *A*, effect of PMA-induced VEGF mRNA expression by antisense (*AS*) oligonucleotide to PKC-α, -β, or -ζ. U373 cells were mock-transfected or transfected with AS oligonucleotide (0.1, 0.5, or 1 μM) to PKC-α, -β, or -ζ. The cells were recovered overnight and treated with 100 nM PMA for 4 h in triplicate. Total mRNA was extracted, and VEGF mRNA level was detected by Northern blot. VEGF mRNA signal was quantified by PhosphorImager analysis and normalized to the 36B4 ribosome-associated mRNA from each lane and calculated as mean ± S.D. The VEGF mRNA from PMA-treated cells were shown as 100% VEGF mRNA levels and compared the mRNA with the control or the AS oligonucleotide-transfected cells. *, significantly different from PMA control ($p \leq 0.05$) by Student's t test. *B*, representative Northern blot of PKC-α or -ζ AS oligonucleotide suppression of PMA-induced VEGF mRNA expression. The mock-transfected cells without PMA treatment (*C*) and with PMA treatment (*P*) were used as controls. Fold induction of VEGF mRNA over mock-transfected control (*C*) was calculated after normalization to the ribosome-associated protein, 36B4, from each lane (*Fold*).

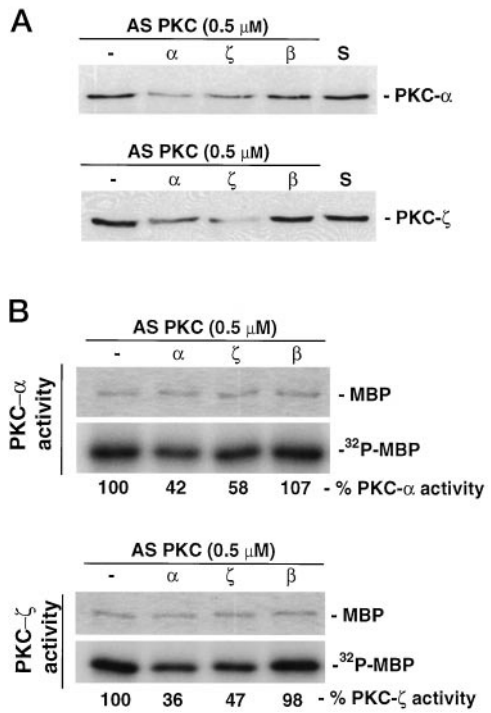

FIG. 8. **Cross-inhibition of PKC isoform synthesis and activity by antisense oligonucleotide to PKC-α or PKC-ζ.** *A*, representative Western blot of PKC-α and -ζ synthesis after PKC-α, -β, or -ζ AS oligonucleotide transfection. U373 cells were either mock-transfected (−) or transfected with 0.5 μM PKC-α, -β, or -ζ AS-oligonucleotide (*AS*). Total cellular proteins were extracted and detected PKC-α or -ζ protein levels by Western blot. *S* indicates standard PKC-α or -ζ from Jurkat cell lysate. *B*, representative *in vitro* PKC-α and -ζ activity after PKC-α, -β, or -ζ AS oligonucleotide transfection. PKC-α or -ζ was immunoprecipitated from mock-transfected (−) or 0.5 μM PKC-α, -β, or -ζ AS oligonucleotide-transfected cell lysate. The *in vitro* PKC-α or -ζ activity was measured by phosphorylation of the 21-kDa myelin basic protein (*MBP*), and ^{32}P-MBP was measured after separation in 12% SDS-PAGE. MBP and ^{32}P-MBP signals were quantified by densitometer and PhosphorImager analysis, respectively. Percent PKC activity over mock transfection was calculated after normalization to the Coomassie Blue-stained MBP from each lane.

PKC-α and -ζ AS-oligonucleotide also showed concomitant decreases in PKC-α activity. PKC-α specific activity was 42 and 58% over mock-transfected control with PKC-α and -ζ AS oligonucleotide transfection, respectively. In a similar manner, PKC-ζ activity levels were 36 and 47% of mock-transfected control with PKC-ζ and -α AS oligonucleotide transfection, respectively. Although the specific isoform oligonucleotides were most effective, the alternative isoform oligonucleotides were still fairly efficient. These results suggest that PKC-α and -ζ AS oligonucleotides cross-inhibit their protein synthesis, indicating that these PKC isoforms may be under strict coordinate control *in vivo*. Since the AS oligonucleotide sequences of the PKC-α and -ζ do not show significant homology, the cross-inhibition of their protein synthesis is likely to be dependent on isoform cross-talk as opposed to nonspecific interactions.

To measure whether blocking of PKC-α and -ζ synthesis through AS oligonucleotides will also affect PMA induction of VEGF mRNA stability, we transfected U373 cells with AS oligonucleotides and analyzed the PMA induction of VEGF mRNA half-life. As expected, a prolonged VEGF mRNA half-life of 3 h was demonstrated with PMA-treated/mock-transfected U373 cells (Fig. 9). In contrast, PKC-ζ AS oligonucleotide-transfected cells completely prevented the PMA induction of VEGF mRNA stability with an observed VEGF mRNA half-life of 0.7 h, which is similar to control VEGF mRNA half-life without PMA treatment (see Fig. 5). Transfection with PKC-α AS oligonucleotide also showed strong suppression of PMA-induced VEGF mRNA stability (data not shown). Taken together, these results suggest that repression of PKC-α or -ζ protein synthesis through AS oligonucleotide transfection can effectively block PMA induction of VEGF mRNA stability.

PKC-ζ Overexpression Up-regulates VEGF mRNA Expression—To determine whether PKC-ζ itself can up-regulate VEGF mRNA expression, we overexpressed PKC-ζ in U373 cells using a cytolomegalovirus enhancer driven FLAG-epitope-tagged PKC-ζ expression vector (18). To ensure that the FLAG-PKC-ζ protein was being expressed after transfection, we used Western blot to detect cytoplasmic PKC-ζ protein levels. As shown in Fig. 10*A*, transfection with the FLAG-PKC-ζ vector resulted in the detection of a higher molecular weight PKC-ζ protein than the standard PKC-ζ, and a substantial increase in FLAG-PKC-ζ protein when compared with the control vector. To evaluate PKC-ζ activity after FLAG-PKC-ζ transfection, we employed an *in vitro* kinase assay (Fig. 10*B*). As expected, PKC-ζ activity correlated to FLAG-PKC-ζ expression when compared with the control cells (2.9-fold increase). In Northern blot analysis, the PKC-ζ overexpressing cells demonstrated

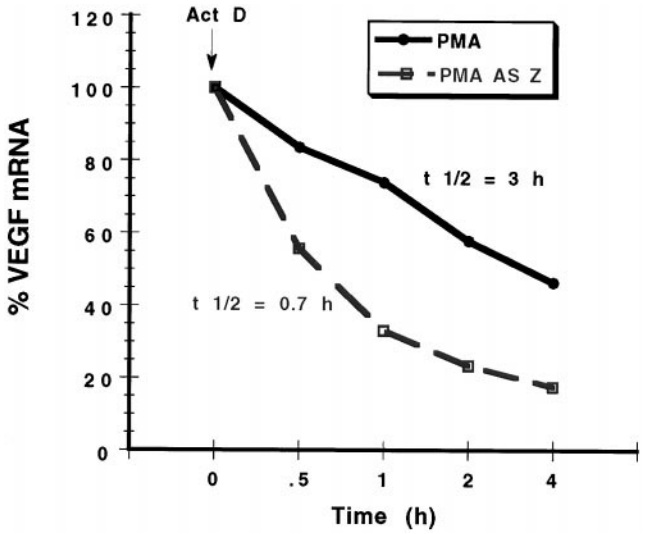

FIG. 9. **PKC-ζ antisense oligonucleotide blocks PMA-induced VEGF mRNA stability.** U373 cells were either mock-transfected or transfected with 0.5 μM PKC-ζ AS-oligonucleotide. The cells were then treated with PMA for 3 h prior to addition of actinomycin D (5 μg/ml) for the indicated times. Total mRNA was then extracted, and VEGF mRNA level was detected by Northern blot. VEGF mRNA signal was normalized with the ribosome-associated mRNA, 36B4, from each lane. VEGF mRNA signal with 3 h of PMA treatment was defined as 100% and calculated % VEGF mRNA remaining after actinomycin D treatment.

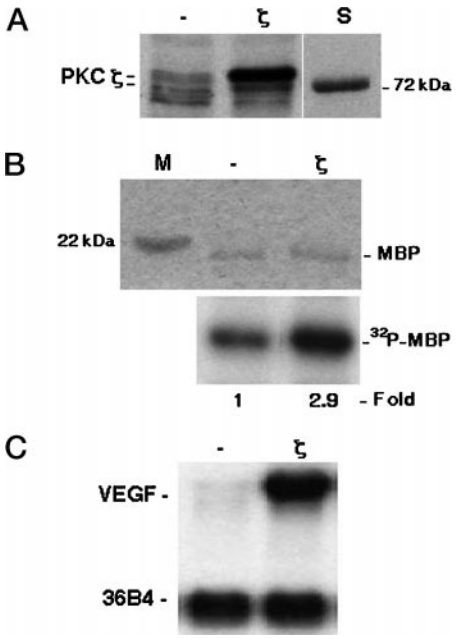

FIG. 10. **PKC-ζ overexpression up-regulates VEGF mRNA expression.** U373 cells were either transiently transfected with empty vector alone (−) or with FLAG-PKC-ζ overexpression vector (ζ). *A*, Western blot detection of PKC-ζ protein expression level in transfected cells. *B*, PKC-ζ activity in empty vector (−) and PKC-ζ overexpression in vector-transfected cells. *C*, Northern blot detection of VEGF mRNA expression in transfected cells. *M* indicates 22-kDa protein marker. *S* indicates standard PKC-α or −ζ from Jurkat cell lysate.

very high levels of VEGF mRNA expression without requiring PMA activation (Fig. 10*C*). This result further provides evidence that PKC-ζ is a major mediator of VEGF mRNA expression in U373 cells. To address whether overexpression of PKC-ζ was affecting VEGF mRNA transcription, we performed cotransfection of the PKC-ζ vector with the VEGF promoter-luciferase reporter construct. We observed no transcriptional up-regulation of luciferase activity from 4 to 36 h after transfection (data not shown). Thus, the high level of steady-state VEGF mRNA expression produced in PKC-ζ-expressing cells was not mediated through transcriptional mechanisms.

DISCUSSION

The tumor promoter, phorbol 12-myristate 13-acetate (PMA), has been shown to induce angiogenesis in mouse skin *in vivo* (23) and up-regulates VEGF expression in many cell types *in vitro* (2, 6–8). In this study, we show that induction of VEGF expression by PMA treatment and PKC activation in human U373 glioblastoma cells is mediated through mRNA stabilization as opposed to transcriptional activation. Antisense oligonucleotide transfection and PKC-ζ overexpression studies directly demonstrated that both PKC-α and -ζ are involved in the regulation of VEGF mRNA stability. Given the fact that most cells respond to hypoxic stimuli by activating both transcriptional and post-transcriptional mechanisms to increase VEGF mRNA expression, this study identifies for the first time that the PKC pathway is a direct activator of VEGF mRNA stabilization independent of transcriptional events.

Diacylglycerol (DAG) binding to a specific site on cPKC and nPKC isoforms induces translocation of PKCs from the cytosol to the cellular membrane, where the enzyme interacts with membrane phospholipids that are required for kinase activity (4, 5). As DAG analogues, phorbol esters are potent activators of cPKC and nPKC isoforms (4, 5). However, chronic treatment with PMA results in the down-regulation of cytosolic and membrane levels of cPKCs and nPKCs (4, 21). On the other hand, aPKCs are not directly activated or depleted by DAG or phorbol esters (21, 24, 25) but can be activated by other lipid intracellular mediators (26–28). There is evidence that PKC-ζ is directly activated by ceramide *in vivo* by treating NIH-3T3 cells with sphingomyelinase C (26). In addition, phosphatidylcholine-derived DAG activates sphingomyelinase C (27). These studies indicate that although DAG is not able to activate PKC-ζ directly, production of ceramide through sphingomyelinase activation will ultimately affect PKC-ζ activity. This indirect activation of PKC-ζ by DAG is further supported by studies that demonstrate that phosphatidylcholine-phospholipase C-derived DAG can activate the mitogen-activated protein kinase pathway through PKC-ζ but not phorbol ester-sensitive PKCs (28). Furthermore, PMA-mediated activation of cPKCs and nPKCs leads to phospholipase A2 activation which, by releasing arachidonic acid from phospholipids, can also activate PKC-ζ (29). Taken together, these studies indicate that although PMA and DAG do not activate PKC-ζ directly, the lipid second messengers produced in PMA/DAG downstream pathways will likely play an important role in PKC-ζ activation which in turn involves post-transcriptional regulation of VEGF mRNA expression.

Depending on PMA dose and treatment time, PKC isoforms, including aPKCs, have shown variable translocation rates to the plasma membrane compartment (29, 30). Recent studies showed that phosphatidylinositol 3-kinase is required for PMA-induced AP-1 activation and cell transformation in JB6 cells (31), and PMA stimulates PIP_3 production in 3T3-L1 adipocytes (32). Accordingly, PIP_3, a phosphorylation product of phosphatidylinositol 3-kinase, potently and selectively activates PKC-ζ (33). Thus, it is possible that PMA-induced PIP_3 will function to recruit PKC-ζ to the cell membrane and activate it. These observations support the view that PMA is modulating aPKCs through lipid second messengers.

Studies have shown that chronic exposure to PMA (24 h), which depletes PKC activity, does not reduce the hypoxia-induced VEGF expression in astrocytes (6). These data provide supporting evidence that VEGF induction can proceed through cPKC- and nPKC-independent pathways. Our data also support this hypothesis since strong and persistent induction of

VEGF mRNA expression (4–8 h PMA treatment) was observed despite PMA reduction of both cPKC and nPKC proteins, thus implicating a role for the aPKCs in VEGF mRNA expression. Furthermore, chemical inhibitors selective to cPKC and nPKC isoforms did not abolish VEGF mRNA expression, and overexpression of PKC-ζ itself strongly up-regulated steady-state VEGF mRNA levels. Combined, these studies indicate that aPKCs, particularly PKC-ζ, are critical mediators involved in VEGF post-transcriptional regulation. Since VEGF mRNA stabilization has been demonstrated to be one of the key mechanisms required for maximal induction of VEGF mRNA in both hypoxic and hypoglycemic conditions, it is interesting to postulate that activation of PKCs, especially PKC-ζ, may be occurring under these conditions. Hypoxic and/or metabolic stress may adversely affect cell membranes resulting in the release of lipid by-products which in turn trigger PKC-ζ activation and ultimately increase VEGF mRNA stability under these conditions. Since VEGF mRNA stabilization occurs after several hours of hypoxic stress (3), involvement of lipid by-products and PKC-ζ activation are even more likely, an interesting possibility we are currently investigating.

Recent studies showed that overexpression of PKC-ζ potentiated PMA-induced PKC activation and suggested a cross-talk between different PKCs (22). Our studies of AS oligonucleotides also suggested that PKCs may cross-regulate their expression and activity through cross-talk. We showed that suppression of PKC-α synthesis by AS oligonucleotides to PKC-α also affected PKC-ζ protein level, and vice versa PKC-ζ synthesis was affected by PKC-α AS oligonucleotides. Thus, it is possible that PKC protein synthesis and total PKC activity are under strict control *in vivo*, and blocking of one PKC isoform synthesis might negatively regulate other PKCs, especially between PKC-α and -ζ isoforms, an interesting hypothesis that is currently under investigation. This cross-talk hypothesis also helps to explain why PKC-ζ protein levels started to decrease after 8 h of PMA treatment in U373 cells (data not shown), a time when PKC-α is decreased to very low levels and VEGF mRNA expression is no longer maintained.

The identification of PKC isoforms as specific modulators of VEGF mRNA stabilization will allow extended investigations into the downstream molecular events that control mRNA-binding proteins or alterations in the mRNA degradation pathways. Thus, defining PKCs as modulators of VEGF mRNA stabilization is a significant advance in understanding the role of signaling pathways that control mRNA metabolism, especially that which potentiates the survival of cells exposed to metabolic stresses or prolonged cytokine activation. Given the data presented here, human tumor cells that are metabolically stressed likely impact the expression of VEGF through intracellular activation of the PKC proteins and PKC-α and -ζ, in particular, which function to increase VEGF mRNA stabilization. Thus, alterations in lipid by-products and PKC-ζ activation is likely an additive mechanism that, when combined with transcriptional increases induced by hypoxia, results in prolonged VEGF expression in tumor cells under stress. These activation pathways will likely proceed until the metabolic stress is relieved by increased vascular permeability and/or neovascularization, or, alternatively, the cells do not survive; thus representing a critical balance in tumor cell survival and progression.

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